IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:		§	
	Yair FELD et al	§	
Serial No.:	09/691,889	§	
Filed:	October 20, 2000	§	Group Art Unit: 1632
For:	Nucleic Acid Constructs And Cells, And Methods Utilizing Same For Modifying The Electrochemical Conductance Of Excitable Tissues.	§	Attorney Docket: **00/20989**
Examiner:	Anne-Marie Falk	§	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF YAIR FELD UNDER 37 CFR 1.132

I am presently employed as CEO of GeneGrafts LTD. I also serve as director at CorAssist Cardiovascular LTD. I received my MD and Ph.D. degrees from the Technion School of Medicine in Haifa, worked as a post-doctoral fellow in the Sonnis Family Research Laboratory for Cardiac Electrophysiology and Regenerative Medicine, Technion School of Medicine in 2003.

My research focuses on excitable tissue modification by cells overexpressing ion channels. Since the beginning of my career, I have published a number of scientific articles in highly regarded journals and books, and have presented my achievements at many international scientific conferences.

I am the inventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official actions issued with respect to the above-identified application.

In this Official action, the Examiner has rejected claims 23, 24, 28-33, 35, 40, 42-46, 48-52, 54-56 and 59-83 under 35 U.S.C. § 112 first paragraph as failing to comply with the enablement requirement.

Appendix A and B enclosed herewith show, while using the teachings of the instant application, in vitro and in vivo experimental support for the treatment of a variety of cardiac and neuronal indications using a number of cells, ion channels and construct systems in well-known appropriate animal models, thus supporting the scope of the claimed invention.

These results conclusively show that the methodology described and claimed in the instant application is enabled, thereby proving that the rejections of claims 23, 24, 28-33, 35, 40, 42-46, 48-52, 54-56 and 59-83 under 35 U.S.C. § 112 first paragraph are unfounded.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

July 5, 2006

Dr. Yair Feld
GeneGrafts Haifa, Israel

Enc:
CV of Yair Feld

Curriculum Vitae

Yair Feld M.D., Ph.D.

Date of birth: 6.12.71

Place of birth: Israel

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Education:

Degree	Subject	University	Graduation	Comments
MD	**Medicine**	Technion – Israel Institute of Technology, School of Medicine, Haifa, Israel	2004	MD graduation thesis award (first place)
Post Doctorate	**Physiology and Biophysics**	The Sonnis Family Research Laboratory for Cardiac Electrophysiology and Regenerative Medicine Technion – Israel Institute of Technology, School of Medicine, Haifa, Israel	2003	Modification of the electrophysiological properties of excitable tissues by cell grafts over expressing ion channels.
PhD	**Physiology and Biophysics**	The Sonnis Family Research Laboratory for Cardiac Electrophysiology and Regenerative Medicine Technion – Israel Institute of Technology, School of Medicine, Haifa, Israel	2003	During the Ph.D. I have demonstrated the possibility of modifying the electrophysiological properties of excitable tissues by genetically modified cell grafts over expressing ion channels.
MSc	**Physiology and Biophysics**	Technion – Israel Institute of Technology, School of Medicine, Haifa, Israel	2000	
BA	**Medical sciences**	Technion – Israel Institute of Technology, School of Medicine, Haifa, Israel	1998	
BSc	**Physics**	University of Tel-Aviv, Faculty of Physics and Astronomy, Israel	1995	Cum laude

Current positions:

2003 – CEO, founder, and director of GeneGrafts LTD. GeneGrafts is a biotechnology company aimed at developing a novel treatment for cardiac arrhythmias, Parkinson's disease, and diabetes mellitus type II by transplantation of autologous cell grafts overexpressing ionic channels. The company received a seed investment of $900,000 US, and following two years of operation another $650,000 US.

2002 – Founder and Member of the board of directors of CorAssist Ltd. CorAssist Ltd is a startup company developing a passive device for the treatment of diastolic heart failure. The company was founded in 2002 based on a patent pending (Patent no. 2 below). CorAssist seed financing was $400,000 US. The company received an investment of $4.5 M in December, 2005.

Previous positions:

2004- Physician, Rambam medical center (night shifts).

2003-2004 - Intern at the Rambam medical center.

1998 – 2002 - Tutor at the Technion faculty of medicine, physiology department.

1996 – 1998 - Physicist in an electro-optics company – Duma-optronics.

1994 – 1995 - Non-frontal tutor at the University of Tel-Aviv Faculty of physics.

1995 - 2002 - Coach of the Technion's squash team.

Publications:

1. **Yair Feld**, Meira Melamed-Frank, Izhak Kehat, Dror Tal, Shimon Marom, Lior Gepstein. Electrophysiological Modulation of Cardiomyocytic Tissue by Transfected Fibroblasts Expressing Potassium Channels: A Novel Strategy to Manipulate Excitability. ***Circulation***. 2002 Jan;105:522-529.
2. Gideon Meiry, Yotam Reisner, **Yair Feld**, Stanislav Goldberg, Michael Rosen, Noam Ziv and Ofer Binah. Evolution of action potential propagation and repolarization in cultured neonatal rat ventricular myocytes. ***J Cardiovasc Electrophysiol***. 2001 Nov;12(11):1269-1277.
3. Lior Gepstein, **Yair Feld**, Lior Yankelson. Somatic gene and cell therapy strategies for the treatment of cardiac arrhythmias. ***Am J Physiol Heart Circ Physiol*** 2004 Mar, 286(3):H815-22.
4. Lior Yankelson*, **Yair Feld***, Shimon Marom, Lior Gepstein. Cell Therapy

for Modification of the Myocardial Electrophysiological Substrate. ***Submitted.***

(* - equal contribution)

5. **Yair Feld**, Shay Dubi, Yotam Reisner, Ehud Schwammenthal, Amir Elami. Future strategies for the treatment of diastolic heart failure. *Acute Cardiac Care. 2006; 8: 13-20*

Grant Supports:

1. Hydrostatic pressure reduction in the kidney - Chief scientist office (2006) – Tnufa program $15,000.
2. Modification of excitable tissues by genetically engineered cellular grafts. State of Israel – Chief scientist office (2000)– Nofar program $100,000.
3. Genetically engineered cell grafts transfected with ionic channels: A novel cell therapy strategy for treatment of abnormalities in excitable tissues. Mitchel foundation (1999) $25,000.

Awards:

2001 – J.J. Kellerman young investigators award – first place on behalf of the Israel Cardiology Association.

2003 – Hershel Rich – Technion Innovation Award.

2004 – MD graduation thesis award (first place).

Selected abstracts:

1. BIOMED 2006, Israel. GeneGrafts. **Yair Feld**
2. BIO 2006, Chicago. Somatic Cell and Gene Therapy for treating chronic cardiac disease: Technology and Challenges. **Yair Feld (presenter).**
3. Israel Society for Gene Therapy. MODIFICATION OF NEURAL NETWORK DISCHARGE BY TRANSPLANTATION OF GENETICALLY MODIFIED FIBROBLASTS OVEREXPRESSING ION CHANNELS. T Bresler, D Shalhevet, R Miary, L Gerion, L Gepstein., S Marom, **Y Feld (presenter).** 2006.
4. Israel Society for Gene Therapy. Cardiac electrophysiological modification by fibroblasts overexpressing ion channels. Z Gluzman, M Boulos, R Miary, T Bresler, L Yankelson, L Gepstein, S Marom, **Y Feld**. 2006.

5. International Society for Heart Research. AV node modification for the treatment of atrial fibrillation by transplantation of fibroblasts overexpressing potassium channels. **Yair Feld (presenter)**. 2006.
6. European Society of Cardiology Congress. Transfected Fibroblasts Modulate Cardiac Excitability. **Yair Feld (presenter)**. 2005.
7. American Society of Gene Therapy 8th Annual Meeting. Refractory Period Prolongation by Fibroblasts Overexpressing the Mutant Kv1.3 H401W Channel in Computer Simulation and Pig Hearts. Shabtay A, Bresler T, Yankelson L, Gepstein L, Marom S, and **Feld Y (presenter)**. 2005.
8. American Heart Association. Cell therapy for modification of the myocardial electrophysiological properties. Lior Yankelson, **Yair Feld**, Lior Gepstein.2003.
9. North American Society of Pacing and Electrophysiology. Assessment of hybrid cardiomyocytic cultures with fibroblasts transfected with potassium channel Kv1.3: A novel approach for gene therapy. **Yair Feld (presenter)**, Meira Melamed Frank, Leonid Heimovitz, Shimon Marom, Lior Gepstein. 2001.
10. International Society for Heart Research. Evolution of action potential propagation and repolarization in cultured neonatal rat ventricular myocytes. Meiry G, Reisner Y, **Feld Y**, Goldberg S, Rosen M, Ziv N, and Binah O. 2001.
11. International Society for Heart Research. Assessment of hybrid cardiomyocytic cultures with fibroblasts transfected with potassium channel Kv1.3: A novel approach for gene therapy. **Yair Feld (presenter)**, Meira Melamed Frank, Leonid Heimovitz, Shimon Marom, Lior Gepstein. 2001.
12. North American Society of Pacing and Electrophysiology. Evolution of action potential propagation and repolarization in cultured neonatal rat ventricular myocytes. Binah O, Meiry G, Reisner Y, **Feld Y**, Goldberg S, Ziv N, and Rosen M. 2001.
13. Israel Heart Society. Assessment of hybrid cardiomyocytic cultures with fibroblasts transfected with potassium channel Kv1.3: A novel approach for gene therapy. **Yair Feld (presenter)**, Meira Melamed Frank, Leonid Heimovitz, Shimon Marom, Lior Gepstein. 2001.

14. International Society for Heart Research. A novel in-vitro model for slow conduction in the human heart: High-Resolution, electrophysiological assessment of human embryonic stem cell derived cardiomyocytes. Kehat I, Druckmann M, Gepstein A, **Yair Feld**, Amit M, Karsenti D, Itskovitz-Eldor J and Lior Gepstein. 2001.
15. American Heart Association. Long Term, High-Resolution, Electrophysiological Assessment of Human Embroynic Stem Cell Derived Cardiomyocytes: A Novel in Vitro Model for the Human Heart. Izhak Kehat, Dorit Karsenti, Michal Amit, Mirit Drukman, **Yair Feld**, Joseph Itskovitz-Eldor, Lior Gepstein. 2000.

Patent applications:

1. Title: Nucleic acid constructs and cells, and methods utilizing same for modifying the electrophysiological function of excitable tissues. Inventors: **Yair Feld**, Lior Gepstein, Shimon Marom, Meira Frank. (US Patent application No. 09/691,889, 10.2000).
2. Title: In-vivo method and device for improving diastolic function of the left ventricle. Inventor: **Yair Feld**. (U.S. Provisional Patent Application 11.2001).
3. Title: Device for selective injection to small arteries by catheterization. Inventor: **Yair Feld**. (U.S. Provisional Patent Application 8.2004).
4. Title: Excitable tissue electrophysiological modulation by transplantation of dermal fibroblasts. Inventors: Tal Bresler and **Yair Feld**. (U.S. Provisional Patent Application 3.2005).
5. Title: METHODS AND DEVICE FOR REDUCING HYDROSTATIC PRESSURE IN THE URINARY SYSTEM. Inventor: **Yair Feld** (U.S. Provisional Patent Application 5.2005).
6. Title: METHODS AND DEVICE FOR THE TREATMENT OF OBESITY. Inventor: **Yair Feld** (U.S. Provisional Patent Application 5.2005).
7. Title: POTASSIUM CHANNEL HUMAN KV1.3-H399W MUTAGENESIS. Inventors: Tal BRESLER, **Yair FELD**, Shimon MAROM, Reem MIARY (U.S. Provisional Patent Application 12.2005).

Military service:

Reserve: Rank: Lieutenant. Position: Physician. Unit: Infantry.

Obligatory: 1990-1993 - Position: Officer of manpower. Unit: Infantry.

General:

Selected achievements as a squash player:

- Champion of the Israeli national championships for juniors in the years 1989-1990.
- Champion of the Dutch junior open 1989 – first Israeli to win an international tournament in squash.
- Member of the Israeli national team in the European team championship for juniors 1989.
- Captain of the Israeli national team and first rank on it behalf in the European team championship for juniors 1990.
- Member of the Israeli national team in the European team championships for men in the years 1996-2000.
- Captain of the Israeli national team in the European team championships for men in the years 1999, and 2000.
- Runner-up in the Israeli national championship for men 1998.
- Third place in the Israeli national championships for men in the years 1989, 1990, 1999, and 2000.
- Sportsmanship award in the European team championship for men 2000.
- Sportsmanship award from the Technion student's association 2002.
- Member of the Israeli National Team in the Maccabiah games 12-17.

APPENDIX A

Refractory Period Prolongation in Pig's Hearts Implanted With Fibroblasts Overexpressing the Mutant Kv1.3 H401W Channel

Prolongation of the refractory period is one of the different strategies for the treatment of cardiac arrhythmias. The Kv1.3 channel expresses a long tail current that renders it attractive as a potential modulator of cardiac refractory period when overexpressed by cardiomyocytes or other which can electrically couple to cardiomyocytes. It was previously demonstrated that Kv1.3 channel in which the histidine at position 401 was replaced by tryptophan (Kv1.3 H401W) introduced a rapid inactivation property to the channel.

The following results substantiate the possibility of prolonging the refractory period of pig hearts at specific loci by transplanting fibroblasts overexpressing various ion channels, such as Kv1.3 and its mutant and Kir2.1. These findings directly support the use of the claimed technology for treating atrial fibrillation and ventricular tachycardia.

Materials and Experimental Procedures

Expression systems - The NIH 3T3 fibroblast cell line that was stably transfected to express the voltage gated potassium channel Kv1.3 was generated as described in the instant application (see Example 5 of the instant application). Briefly, stable transfection of the plasmid pRC/CMV/Kv1.3 in fibroblasts was achieved using electroporation with a single pulse of 200 V, 960 μF followed by antibiotic selection of Neomycin resistant clones with 400 μg/mL of G-418 (Gibco). Colonies were picked and tested for channel expression using whole-cell patch clamp recordings (see page 43 second paragraph of the instant application).

A Kv1.3 channel in which the histidine at position 401 was replaced by tryptophan (Kv1.3 H401W) was generated using a standard site directed mutagenesis procedure using rat Kv1.3, as described in length at Appendix B.

NIH 3T3 cells were transfected to express the Kv1.3 H401W using Lipofectin Reagent (Invitrogen) according to manufacturer's instructions. Seventy two hours following transfection, 500μg/ml of G-418 (GIBCO) were added to the growth medium

for clonal selection of neomycin resistant cells. Clones were isolated using cloning rings. Cells were grown in DMEM, supplemented with 10 % FCS, Gentamycin (50μg/ml) and Penicillin (100 units/ml) and Streptomycin (100μg/ml).

NIH 3T3 were also transfected to express the Kir 2.1-HIS tag pReciever-MO1a with CMV promoter (described in Page 6 line 2 of the instant application) using the lipofectin Reagent (Invitrogen) as mentioned above.

Control NIH 3T3 expressing exogenous GFP plasmid pIRES2-EGFP with CMV promoter were generated as described in Appendix B.

Electrophysiological recordings - Whole-cell patch clamp recordings were conducted at room temperature using Axopatch 200 (Axon Instruments) as described in the instant application (see Example 5). Data were collected using a PC computer with pClamp 9.0 software (Axon Instruments), low-pass filtered at 5 to 10 kHz and sampled at 20 kHz. Electrodes for voltage-clamp experiments were made from fire-polished aluminum silicate glass, with a resistance of 2 to 3 MΩ. The pipette solution contained (in mmol/L) KCl 140, Na2ATP 10, EGTA 10, HEPES 5, CaCl2 1, and MgCl2 1 (pH 7.3). The bath solution contained (in mmol/L) NaCl 140, KCl 3, HEPES 10, glucose 10, MgCl2 2, and CaCl2 2.

In vivo animal study (described in Example 1; and Page 58 last paragraph and page 59 lines 1-15 of the instant application).

Ventricular ERP modification - Six domestic pigs (20-30 kg) were studied. Anesthesia was induced with 5-10 ml of intravenous sodium pentothal 2.5 % solution and maintained with inhaled isoflurane 2 % in oxygen. Following a right thoracotomy, the heart was exposed and a total of 6 x 10^6 cells (H401W transfected NIH 3T3) were transplanted at a total of six different locations in the anterior free wall of the right ventricle. The sites of cell transplantation were marked with a suture. To prevent graft rejection, the animals were treated with an immunosuppressive regimen (cyclosporine 10mg/kg/d, methylprednisolone 3mg/kg/d, and azathioprime 50mg/d). Seven days following cell transplantation, a detailed electrophysiological study was performed with induction of ventricular tachycardia by rapid pacing.

AV node modification - Four domestic pigs (20-30 kg) were studied. Anesthesia was induced as described above. The jugular vein was catheterized and an activation map

of the right atria was performed using the Noga star catheter and the Carto system (Biosense Webster). A total of 50 x 10^6 cells transfected with the mutant Kv1.3 H401W were transplanted at a five different injections where His recording was positive. To prevent graft rejection, the animals were treated as described above. Seven to ten days and 14-20 days following cell transplantation, a detailed electrophysiological study was performed.

Ventricular ERP - The electrophysiological study included a standard ventricular pacing protocol to determine the local myocardial effective refractory period (ERP). The ERP was evaluated by applying a train of 20 stimuli at a fixed cycle-length of 350 ms (S1), followed by a single premature stimulus (S2). The S1-S2 coupling interval was gradually shortened. The ERP was determined as the shortest S1-S2 coupling interval still capable of eliciting a ventricular response. Stimuli were generated using a computer controlled STG 1004 stimulus generator with the matching MC-Stim software (Multi Channel Systems, Reutlingen, Germany).

The ERP was measured at the site of cell transplantation and at remote sites, seven days following cell grafting.

AV node ERP – was affected as described above. In both ventricular ERP and AV node ERP measurements, the ERP was measured also following an intravenous application of 9 μg/kg of Margatoxin (Alomone Labs, Jerusalem, Israel), a specific Kv 1.3 channel blocker, in order to dissect the role of the overexpressed channel in altering the myocardial electrophysiological properties,. In two cases atrial fibrillation was induced at baseline and 10 days following transplantation by burst atrial pacing.

Statistics - All in vivo results are expressed as mean ± SEM. Data was compared using either paired or unpaired t-test with Bonferoni correction.

Results

Voltage clamp analysis of the transfected fibroblasts - Wilde type (WT) Kv1.3 and mutant Kv1.3 H401W were expressed in NIH 3T3 fibroblasts. Once stable clones were identified and isolated, cells were clamped to -100 mV and K+ currents were elicited by a 500 ms step of +10 mV. The mutant Kv1.3 H401W expressed a fast inactivation compared to the WT Kv1.3 channel (Figures 1C and 1A respectively). Tail currents were measured at -75 mV step +5mV up to -35 mV following a 100 ms step to

40 mV. The long tail currents phenotype of the mutant Kv1.3 H401W compared to the WT Kv1.3 is demonstrated in Figures 1D and 1 B, respectively. The inactivation properties of the WT Kv1.3 channel have been described in the instant application, see Example 5. The rate of transition from open to inactivated state was γ=0.0014 $msec^{-1}$, and the rate of transition from inactivation to open state was δ=0.0001 $msec^{-1}$. In the mutant Kv1.3 H401W the calculated rate of transition from open to inactivated state was γ=0.005 $msec^{-1}$, and the rate of transition from inactivation to open state was δ=0.0074 $msec^{-1}$.

In vivo studies

Ventricular ERP - The effective refractory period (ERP) was assessed at the area of transplantation of the fibroblasts overexpressing the mutant Kv1.3 H401W channel, the WT Kv1.3 channel and at remote site as a control. The ERPs were recorded 7 days following transplantation to allow functional integration of the cells as demonstrated by immunohistological studies (Figure 2). Western blot analyses were conducted to confirm overexpression of the Kv1.3 and the Kir2.1 channels (Figure 2). The measured ERPs were 204±15 ms (n=21) at the control area, 223±28 ms (n=11) at area transplanted with fibroblasts overexpressing GFP, 227±15 ms (n=3) at area transplanted with fibroblasts overexpressing the WT Kv1.3 channels, 259±22 ms (n=7) at area transplanted with fibroblasts overexpressing the mutant Kv1.3 H401W channel and 260±18 ms (n=7) at area transplanted with fibroblasts overexpressing the Kir2.1 channel (Figure 3). These results show that both wild type Kv1.3 and Kv1.3 H401W expressing cells can be used to prolong ventricular ERP.

AV node modification - The effective refractory period (ERP) was assessed at the AV node area by applying stimuli to the atria and measuring the ventricular respond. Only fibroblasts overexpressing the mutant Kv1.3 H401W channel were transplanted. The ERPs were recorded at baseline (n=4), 7-10 days following transplantation (n=4) and again 14-20 days following transplantation (n=2), to allow functional integration of the cells as demonstrated by immunohistological studies (Figure 2). The measured ERPs were 270 ± 18 ms (n=4) at baseline, 297.5 ± 15 ms (n=4) 7-10 days following fibroblasts transplantation, 272.5 ± 14 ms (n=4) following margatoxin application, 14 to 20 days following transplantation ERP increased to 281.5 ± 3 ms (n=2) and following margatoxin

application reduced to 225 ± 26 ms (n=2) (Figure 4). These results demonstrate the feasibility of this strategy for a rate control treatment for atrial fibrillation.

Induction of acute atrial fibrillation - In two pigs acute atrial fibrillation was induced by burst atrial pacing at baseline and 7-10 days following fibroblasts transplantation. The ventricular response was reduced by an average of 41%. Figure 5 demonstrates representative traces at baseline and following fibroblasts transplantation. These results clearly support the use of the cells of the present invention for treating atrial fibrillation.

Figure Legend

Figure 1: A. Voltage clamp recording from WT Kv1.3. B. Tail current recorded from WT Kv1.3. C. Voltage clamp recording from mut Kv1.3 H401W (lower) transfected NIH/3T3 fibroblasts performed at holding potential of -100mV. D. Tail current recorded from Mut H401W.

Figure 2: Western blot analysis of Kv1.3 (panel A) and Kir2.1 (panel B). Total heart proteins from sites of implanted cell grafts as well as from control sites were extracted, separated by SDS-PAGE and immunoblotted with anti-Kv1.3 or anti-Kir2.1. Molecular weights of peptides are shown left to each panel. Lane 1, 2: non-implanted locations, lanes 3-5: locations implanted with Kv1.3 expressing cells,
lane 6: location implanted with Kir2.1 expressing cells, lane 7: negative control – rat skin fibroblasts, lane 8: positive control – rat skin fibroblasts transfected with Kv1.3 fused to GFP, lane 10: location implanted with Kir2.1 expressing cells.
C-F Immunohistochemical detection of cell grafts expressing His-tagged Kir2.1. Heart slices from sites of implantation were cut by microtome into 7 micron preparate slides and probed with heart marker (Troponin1) or anti-poly-His antibody. (C) Implanted cells overexpressing the ion channel KIR2.1 (contains poly-Histidine tag) labeled with anti-poly-Histidine. (D) Heart tissue labeled with anti-Troponin1. (E) DIC image of the tissue. (F) Merged image of A and B, Green- cell grafts; Red- heart tissue.

Figure 3: Cardiac ventricular electrophysiological modulation in pigs: control area, fibroblasts overexpressing GFP, Kv1.3 potassium channel, Mut Kv1.3 H401W, and Kir2.1.

Figure 4: A shows activation map of the right atria using the Carto system. Pink ball marks the bundle of His. B. shows the AV node effective refractory period was increased by 11 ± 5% (n=4) 7-10 days following transplantation that was reversible following application of Margatoxin (MTX – a specific blocker of Kv1.3 channel). 14-20 days following transplantation the ERP was increased by 18 ± 8% (n=2) compared to baseline and was reduced to 93 ± 18% of baseline following MTX administration.

Figure 5: A representative ECG trace during acute atrial fibrillation at baseline (upper trace) and 10 days following fibroblasts transplantation (lower trace). The ventricular respond was reduced from 170 Beats Per Minutes (BPM) at baseline to 100 BPM following fibroblast transplantation.

APPENDIX B

In-vitro and in-vivo modulation of Neural Network Discharge by Transplantation of Genetically Modified Fibroblasts Overexpressing Ion Channels

Modified fibroblasts expressing the mutated potassium channel Kv1.3:H401W and the gap junction protein Connexin36 were used to modulate the electrical activity of neural networks both *in-vitro* and *in-vivo*. First, the ability of modified fibroblasts to create gap junctions with the neural network using a dye coupling technique was tested. Thereafter, these cells were shown to modulate the electrical activity of neural networks *in-vitro* using co-cultures of cortical neurons and modified fibroblasts. The modified fibroblasts were stereotaxically implanted into the GPi and SNr of hemiparkinsonian rats, resulting in the reduction of parkinsonism-like rotational behavior.

Together, these results suggest that modified fibroblasts can be used to electrically couple with the neural tissue and more importantly can modify its electrical activity.

Material and Experimental Procedures

(See Example 3 and Example 4 of the instant application)

In vitro experiment:

Preparation of cultured hippocampal neurons - Primary cultures of dissociated rat hippocampal cells were prepared as previously described (Ryan *et al*, 1993). To that end, hippocampal CA1-CA3 regions were dissected from 1-2 day old Sprague-Dawley rats, dissociated by trypsin treatment followed by triturization with a siliconized Pasteur pipette and then plated onto coverslips coated with Poly-D-Lysine (Sigma, USA) inside 8 mm diameter glass cylinders (Bellco Glass, NJ, USA) microwells. Culture media consisted of minimal essential media (Gibco BRL, MD, USA), 0.6% glucose, 0.1g/l bovine transferrin (Calbiochem CA, USA), 0.25g/l insulin (Sigma), 0.3g/l glutamine, 5-10% FCS (Sigma), 2% B-27 supplement (Gibco) and 8μM cytosine b-D-arabinofuranoside (Sigma). Cultures were maintained at 37°C in a 95% air, 5% CO2 humidified incubator, and culture media was replaced every 7 days.

Preparation of cultured cortical neurons - Primary cultures of rat cortical neurons were prepared as described previously (Eytan et al. 2003; Marom and Shahaf 2002; Shahaf and Marom 2001). Briefly, cortical neurons were obtained from newborn rats within 24 h of birth. The cortex tissue was enzymatically digested and

mechanically dissociated, and the neurons were plated directly onto substrate-integrated multi-electrode array (MEA) dishes (Gross 1979; Stenger and McKenna 1994). Cultures were grown in MEM supplemented with heat-inactivated horse-serum (5%), glutamine (0.5 mM), glucose (20 mM), and gentamycin (10 μg/ml), and maintained in an atmosphere of 37°C, 5% CO_2–95% air, in a tissue culture incubator and during the recording phases. Experiments were performed during the third week after plating, following the period of functional and structural network maturation.

Electrophysiological measurements - Commercially available arrays of 60 Ti/Au/TiN electrodes, 30 μm diam, spaced 200 μm from each other were used (MCS, Reutlingen, Germany). The insulation layer (silicon nitride) was pretreated with poly-L-lysine. The arrays were connected to a 60-channel amplifier (B-MEA-1060, MCS), with frequency limits of 1–5,000 Hz and a gain of x1,024, which was further amplified using *MCPPlus* variable gain filter amplifiers (Alpha-Omega, Nazareth, Israel). Data was digitized, using two parallel 5200a/526 A/D boards (Microstar Laboratories). Each channel was sampled at a frequency of 24 ksample/s and output was prepared for analysis using the AlphaMap interface (Alpha Omegal). Thresholds (x8 RMS units—typically in the range of 10–20 μV) were defined separately for each recording channel prior to the beginning of the experiment. All data presented herein was obtained from threshold crossing events. Analysis of sample experiments revealed that the results were not qualitatively affected by passing the data through a spike-sorting procedure (principal component methodology; AlphaSort software, Alpha Omega).

Several records were performed under the application of a Kv1.3 channel blocker (rCarybdotoxin; Alomone aboratories). The blocker was applied to the array, using a standard perfusion system, to a final concentration of 1nM.

DNA constructs and site-directed mutagenesis - pEGFP-C1 was purchased from Clontech (clontech cat# PT3028-5), rat connexin36-EGFP pEGFP-N3 with CMV promoter was received from Dr. Zoidl G., and hKir2.1 was purchased from Genecopoeia (EX-A0606-M01) pReciever-MO1a with CMV promoter. Mutatgenesis of wild type Kv1.3 channel (rKv1.3:H401W) was effected as follows: Wild type rat KV1.3 was received from Prof. Steve Golstein. The tryptophane to histidine exchange at position 401 was generated by cloning the wild type rat KV1.3 into the pGEM cloning vector, containing the endogenous Acc1 restriction site and the designed BamH1 restriction site on both sides of the desired mutation location (position 401).

Primers used creating the mutation:

rKv3(41-3) s Acc1:

5' TTC ATT GGG GTC ATC CTT TTC TCC AGT GCA GTC TAC TTT GCT GAG 3'

rKv3(41-3) as1 BamH1:

5' AAG GGA TCC CAC AAT CTT GCC TCC TAT GGT CAC TGG CCA CAT ATC ACC ATA 3'

The mutated 174 base-pair fragment was obtained and ligated into a PCR cloning kit (Qiagen®; Cat. No:231122). Positive E-coli colonies were mini prepped and sequenced. The wild-type fragment was then exchanged with the mutated fragment using the Acc1 and BamH1 restriction sites. Positive colonies were again sequenced. The exchanged Kv1.3 gene (referred to as H401W-1) was amplified with the following primers, each containing the designed restriction sites:

rKv3 (41-3) s + Bgl2:

5' CAT AGA TCT GAA TCG GAG TGA GTG CCG 3'

rKv3 (41-3) as + EcoR1:

5' ATG AAT TCC ACA CAA TAC TGG GCA CAG A 3'

Finally, the 1.7 kb mutated fragment was cloned into pIRES2:GFP vector (clontech PT3267-5).

Cell lines - All cell lines were created using the same method: NIH/3T3 fibroblasts were transfected with the selected DNA plasmid using Lipofectin Reagent (Invitrogen) and standard transfection protocol as recommended by the manufacturer. 72 hours after transfection, 500μg/ml of G-418 (GIBCO) was added to the growth medium for selection of cells expressing the neomycin resistance gene. Clones were isolated using standard cloning rings (corning). Cells were grown in DMEM, supplemented with 10% FCS, Gentamycin (50μg/ml) and Penicillin (100 units/ml) - Streptomycin (100μg/ml). Medium was replaced every 3 days.

Dye coupling - To monitor the diffusion of fluorescent molecules through gap junctions (dye coupling) the method described by Tomasetto *et al.* (1993) and Goldberg *et al.* (1995) was used. See also reference number 2 of the instant application. Briefly, cells were preloaded with 2 different dyes: the gap junction permeant dye Calcein-AM (using the acetoxymethyl ester derivative of the dye), and the membranal dye DiD (Molecular probes). The loaded cells were then sown over a

monolayer of unloaded cells. Within a short time, fluorescent gap junction permeant dye was observed passing from "donor" to "acceptor" cells.

Loading procedure: 5μM calcein-AM (Fluka17783) and 2.5μM DID (Molecular probes) were added to the medium. Cells were incubated with the dyes at 37°C for 30 minutes. Excess unincorporated dye was removed by three consecutive washes in PBS+2mM ca^{2+} and 2mM Mg^{2+}. Labeled cells were harvested using standard trypsinization methods and centrifuge for 3 minutes in 2000rpm. Cell pellet was resuspended in serum-containing medium. A small number (~8 $\times 10^3$) of the calcein and DiD-loaded cells (donor cells) was added to the monolayer of cultured fibroblasts or cultured neurons (acceptor cells). After four hours incubation the co-culture was inspected by microscope for fluorescent intensity in acceptor cells.

Immunohistochemistry (page 52 4th paragraph of the instant application)- Cultures and Co-cultures were grown on cover slips, followed by retrospective immunocytochemistry analysis. Co-cultures were fixed by flooding the chamber with a fixative solution consisting of 4% formaldehyde and 120 mM sucrose in phosphate buffered saline (PBS). The cells were permeabilized for 10 min in fixative solution to which 0.25% Triton X-100 (Sigma) was added. Cells were washed 3 times in PBS, incubated in 10% bovine serum albumin (BSA) for 1 hour at 37°C, and incubated overnight at 4°C with primary antibodies in PBS and 1% BSA. Cells were then rinsed 3 times for 10 minutes with PBS, and incubated for 1 hour at room temperature with secondary antibodies in PBS and 1% BSA. Finally, cells were rinsed with PBS and imaged immediately. Primary antibodies used were: anti-GFP (santa cruz cat# sc8334) and anti-glutamic acid decarboxylased (GAD)(chemicon # MAB351R)

Western blot analysis - Confluent 10cm plates were washed with D-PBS (Dulbecco's Phosphate Buffered. Saline, Biological industries Beit Haemek, cat number 02-023-1A) and cell lysate was obtained using 300μl of cold RIPA buffer (RIPA lyses buffer Santa Cruz Biotechnology, cat number sc-24948). The lysate was centrifuge at 13000g for 10 minutes at 4°C and sample buffer (Bio-rad cat # 161-0737; {beta}-mercaptoethanol was added) was added to supernatant. Sample was incubated for 5 minutes at 95°C, loaded onto a 10% SDS–PAGE (Novex; cat#EC6078BOX) and immobilized to a nitrocellulose filter. The filter was incubated in PBS with 1% casein (Bio-rad cat# 161-0783) for 30 minutes at RT, then probed with anti-Kv1.3 polyclonal antibodies (Chemicon; cat#AB5178) or anti-Cx36 (Zymed; cat#51-6200) overnight at 4°C, followed by goat anti-rabbit horseradish

peroxidase labeling (sigma; cat#A6154). After extensive washing in PBS, bands were visualized by chemiluminescent reaction (Dab, Sigma).

Whole cell patch clamp recordings - Voltage-clamp recordings were performed using the whole-cell patch clamp technique, all measurements were done at room temperature. Patch pipettes (2–4 MΩ) were prepared from glass capillary tubes (Jencons cat#687-055) using a double-stage puller (Narishige pp-830). Currents were recorded with an Axon Instruments amplifier (Axopatch 200), digitized by DigiData 1322A digitizer (Axon instruments). Data was analyzed using pCLAMP software (version 8.0). Bath solution for the whole-cell recordings contained (in mM): 140 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Glucose and 10 HEPES (pH 7.4). Pipette solution for the whole-cell recordings (in mM): 140 KCl, 10 Na_2 ATP, 1 $CaCl_2$, 1 $MgCl_2$, 10 EGTA and 5 HEPES (pH 7.4).

In- vivo experiments

Animals - Adult male Wistar rats (Harlaan, Israel) weighing 200-250g were housed in groups of four in a temperature controlled environment under 12/12 hours light-dark cycle.

6-OHDA lesion procedure - Desipramine HCl (10mg/Kg, s.c.) was administrated 30 minutes prior to the stereotaxic injection of 8μg/4μl 6-OHDA (in saline containing 0.1% ascorbic acid) into the right medial forebrain bundle (MFB). Injection coordinates, relative to the bregma, were: (AP): -4.4; (LR): 1.3; (DV): -7.7 (from dura) or -8.1 (from skull).

Perfusion rate was 1μl /min for 4 minutes. At the end of each injection the needle (30G with round-blunted tip) was left *in situ* for an additional 5 minutes and then slowly retracted.

Cell implantation - $2.5*10^5$ cells (in phosphate buffered saline supplemented with 1mg/ml glucose) were streotaxicaly injected to the GPi. The same amount of cells was stereotaxically injected to the SNr. Coordinates used (relative to bregma adn skull):

medial globus pallidum (MGp) - (AP): -2.8, (LR): +2.9, (DV): -7.7 .

substantia nigra reticulum (SNr) - (AP): -5.5, (LR): +2.3, (DV): -8.0 .

At each site cells were injected over a period of 5 minutes at a rate of 1 μl/min. At the end of the 5 minutes injection period, the needle was left, *in situ*, an additional 5 minutes before being slowly retracted.

Behavioral test - Initially, 20 rats were lesioned with 6-ODHA. 5-6 weeks after 6-OHDA lesion each animal was prescreened for apomorphine-induced rotational asymmetry. Rats were placed in a cylindrical cage and injected with 0.25mg/Kg apomorphine (sigma, cat# a-4393) after a five-minute acclimatization period. Numbers of rotations, both ipsilateral and contralateral, were recorded at five-minute intervals using an automatic rotometer (Rota-Count-8; Columbus Instruments) over a 1.5h period. 12 rats were classified as Parkinsonism after meeting the threshold criteria of seven or more full contra-lateral rotations/minute.

Microdialysis - A hole was drilled through the skull at the following coordinates: A: -2.56, and L: 2.6 relative to Bregma., A guide cannula (CMA/11, cat# 8309018) was inserted through this hole to a depth of 7.7 mm relative to skull, corresponding to the internal globus pallidum, and anchored in place using dental cement. Microdialysis measurements were carried out using an individual free running cave system connected to sample collector. CSF buffer was perfused via a probe, inseted through the guide cannula, at a rate of 1.5µl/min for 20 minutes. Glutamate levels were analyzed by CMA 600 microdialysis analyzer. When glutamate levels in the samples showed stable values, 3 mg/Kg haloperidol, or the specific D2 receptor antagonist Raclopride (3mg/kg) were administrated s.c. Twenty minutes later a second microdialysis measurement was taken.

Results

Connexin36 (Cx36) expressed in NIH3T3 is functional - GABAergic inter neurons in the brain, specifically parvalbumin-contianing neurons, express the gap junction protein Cx36 (Belluardo *et al.* 2000). Therefore, in order to modulate the inhibitory GABAergic neural network, cells of the present invention were modified to expresses Cx36. This protein will enable the fibroblasts to create gap junctions specifically with the GABAergic inhibitory neurons. NIH/3T3 clone, overexpressing the gap junction protein Cx36 merged with GFP (NIH/3T3:Cx36gfp) was prodcued using standard transfection and cloning methods. Protein expression was confirmed using western blot (data not presented) and immunocytochemistry analysis (Figure 1).

The dye coupling method was used to determine the functionality of the exogenous protein. To that end, the amount of dye transferred between donor and acceptor cells (quantified as fluorescent intensity in acceptor cells) was measured in two different sets of co-cultures: (a). In the control co-culture, donor cells were naive NIH/3T3 which were seeded on unlabeled acceptor-monolayers of naïve NIH/3T3,

(b). In the study culture, donor cells were modified fibroblasts (NIH/3T3:Cx36gfp) seeded on unlabeled acceptor-monolayer of modified fibroblasts (NIH/3T3:Cx36gfp).

It is important to note that naïve NIH/3T3 endogenously express gap junction proteins as Cx43 (but do not express Cx36). So, naïve fibroblasts should also be able to transfer dye, but to a smaller extend compared to modified fibroblasts over-expressing the exogenous Cx36 gene.

Thus, the above results show that the amount of dye transferred in the modified fibroblasts co-culture was significantly higher as compared to dye transferred between naive fibroblast co-cultures. The averaged fluorescent intensity of acceptor fibroblasts in naïve co-culture was less then <200 (fluorescent arbitrary units); while the averaged fluorescent intensity of acceptor fibroblasts in co-culture of NIH/3T3:Cx36gfp was greater than 300 (fluorescent arbitrary units) as shown in Figure 2.

These results indicate that the modified fibroblasts generated according to the teachings of the instant application have more functional gap junctions in comparison to naïve fibroblasts, demonstrating that the exogenous protein is functional.

Fibroblasts expressing Cx36 can create gap junctions with neurons - After establishing that NIH:Cx36gfp fibroblasts express functional Cx36, the ability of these modified cells to create gap junctions with neurons in hippocampal cultures was tested.

The ability of NIH:Cx36gfp to create gap junctions with neurons was assessed using the dye coupling technique, in which dye transfer between donor fibroblasts and acceptor neurons is indicative of the creation of effective gap junctions. NIH:Cx36gfp fibroblasts were loaded with calcein AM and membranal dye (DiD) and then seeded on mature hippocampal neuron monolayers. Four hours following fibroblasts seeding a clear fluorescent signal appeared in number of neurons in the culture (Figure 3). This result suggests that modified fibroblasts, expressing the protein x36 are able to create gap junctions with neurons in culture.

Modified fibroblasts can modulate the electrical discharge of cultured neurons - After demonstrating the ability of NIH:Cx36gfp fibroblast to create gap junctions with hippocampal neurons, their ability to modify neural network discharge was examined. To this end, a new fibroblast clone, expressing both the gap junction protein CX36 and the mutated Kv1.3 ion channel Kv1.3:H401W was created. Cells

were tested for the expression of these proteins using both western blot (data are not presented) and patch clamp analysis (Figure 4).

Primary cortical neurons, cultured on multi electrode arrays (MEA) were used for these experiments. Basal activity of three week old cultures was recorded, after which $5*10^4$ fibroblasts were seeded onto the cortical cultures. Study cultures were seeded with fibroblasts expressing both Cx36 and a mutated potassium channel, Kv1.3:H401W (NIH:CX36gfp:Kv1.3:H401W fibroblasts). Control cultures were seeded with NIH fibroblasts expressing GFP.

The mutated Kv1.3 channel was used in the study group due to the long tail current following activation. The tail current produces a large current sink that prevent a second activation. As shown in Appendix A, cells expressing the mutant Kv1.3 are effective in prolonging the local refractory period in pig's hearts (Also, Shabtay et al. *Molecular Therapy.* 2005 May; Abstract). In this manner, fibroblasts expressing the mutated Kv1.3 channel can prolong the refractory period of the neuron and generally reduce its excitability.

After seeding, cultures were returned to the incubator allowing fibroblasts to settle. After 72 hours incubation a second set of electrophysiology recordings was performed and the influence of fibroblasts on the neural network discharge was assessed. Immediately after, a third set of electrophysiological recordings was performed under the application of rCharybdotoxin, a specific Kv1.3 channel blocker.

Both NIH:GFP fibroblasts and the NIH:CX36gfp:Kv1.3:H401W fibroblasts caused an initial decline in the neural network activity. This decline was stable over a period of three days. Upon addition of the specific channel blocker, the network activity was recovered in co-cultures of neurons with NIH:CX36gfp:Kv1.3:H401W fibroblasts, but not those seeded with NIH:GFP.

The decline in network electrical discharges, observed after seeding the cultures with both types of fibroblasts is most likely the non-specific effect of $5*10^4$ cells, probably still dividing. The recovery of electrical activity, obtained solely in the NIH:CX36gfp:Kv1.3 seeded cultures during blocker application is possibly the result of abrupt changes in the electrical properties of the fibroblasts caused by blocker application. The change in fibroblasts electrical properties caused a change in the electrical discharge of the neural network, suggesting that fibroblasts are electrically coupled to the neural network.

These results indicate that fibroblasts can modulate the neural network discharge and that this modulation depends on the fibroblasts electrical properties.

Modified fibroblasts can modulate glutamate levels in-vivo - After demonstrating the ability of modified fibroblasts to modulate neural network discharge *in vitro*, the possibility of modifying the GABAergic inhibitory system in vivo was tested.

To that end fibroblasts expressing the gap junction protein Cx36 and the potassium channels Kir2.1 (NIH:Cx36gfp:Kir2.1) were implanted in the external globus pallidum nucleus (GPi) of rats. One week following cell implantation, glutamate levels in the internal globus pallidum (GPe) of the same rat were sampled by microdialysis. As soon as sample glutamate levels were stabilized, the dopamine receptor antagonist haloperidol, or the specific D2 receptor antagonist Raclopride were administered. Twenty minutes later another measurement was taken. Control groups included rats implanted with either fibroblasts expressing GFP or administered with medium. Table 1 summarizes the experimental design.

Rats which were implanted with NIH:Cx36gfp:Kir2.1 showed significant increase in glutamate levels after administration of the blocker (either raclopride or haloperidol, see Figure 5). In contrast, rats implanted with NIH:GFP, showed significant decrease in glutamate levels following the injection of raclopride. The rat injected with DMEM, showed the same tendency as that of GFP-expressing cells.
Base line levels of glutamate were similar among all examined rats (with the exception of rat2).

The present results indicate that implantation of NIH:3T3:Cx36gfp:Kir2.1 to the external globus pallidum results in a differential response to the D2 receptor antagonist in comparison to NIH:GFP or medium implanted rats. Based on these first indications, the subsequent behavioral experiments on hemiparkinsonian rats was designed as described in the next section.

Table 1 **- implantation conditions for microdialysis experiments**

Rat #	*Type of fibroblasts implanted*	*Number of fibroblasts implanted*
1	NIH:3T3:GFP	$2.5*10^5$
2	NIH:3T3:GFP	$2.5*10^5$
3	NIH:3T3:Cx36gfp:Kir2.1	$2.5*10^5$
4	NIH:3T3:Cx36gfp:Kir2.1	$2.5*10^5$

5	NIH:3T3:Cx36gfp:Kir2.1	$8.5*10^5$
6	Medium	--------

Modified fibroblasts can improve asymmetric rotation behavior of hemiparkinsonian rats The pathophysiology of Parkinson's disease, includes over-inhibition of the thalamus by the main inhibitory nuclei of the basal ganglia. Therefore, if can the excitability of these nuclei can be reduced, using the modified fibroblasts, this may alleviate motor symptoms of PD.

In order to test this hypothesis, two sets of experiments were conducted with two doses 500,000 and 5,000,000 cells (see integration in Figure 7). In the first set twelve 6-OHDA lesioned rats, previously classified as parkinsonian using the apomorphine-induced rotation test, were randomly sorted to two groups. The study group included 5 rats, implanted with NIH:CX36GFP:Kv1.3:H401W fibroblasts. Fibroblasts were implanted into the main inhibitory nuclei of the basal ganglia - the internal globus pallidum (GPi) (250,000 cells) and sabstantia nigra pars reticulate (SNr) (250,000 cells). In the second set the study group included 6 rats, and 2,500,000 cells were impanted in each nucleus.

NIH:CX36GFP:Kv1.3:H401W fibroblasts express the gap junction protein Cx36 which enables them to create gap junctions with Cx36-expressing neurons; they also express a mutated voltage dependent potassium channel Kv1.3:H401W.

The control groups included 7 rats that did not receive any treatment in the first set and 5 rats injected with saline in the second set.

Results low dose:

The sum of rotations during the 15 minute peak is summarized in the following table (table 2). Data refers to 4 and 5 weeks after PD induction, together with two rotation tests at 10 and 18 days, following cell implantation (CI), respectively. Two of the five treated rats (Figure 6a, red squares), but none of the control group rats (Figure 6b), rotated substantially less 10 days after cell implantation, compared to their baseline levels at 4 and 5-weeks post induction.

Table 2 **- sum of rotations during the 15 minute peak**

Sum 15-min max rotation peak					
	Rat	**4wks**	**5wks**	**CI-1**	**CI-2**
Study group	R02	106	113	150	121
	R04	137	177	61	49
	R05	116	130	155	147
	R10	93	108	125	98
	R14	124	170	73	101
Control	R03	77	126	112	116

group	R06	118	116	101	125
	R08	123	149	171	145
	R13	165	164	132	130
	R18	38	133	111	137
	R19	140	148	130	194
	R20	107	123	131	119

Results high dose All the rats in the study group demonstrated reduction in parkinsonian like rotational behavior to 21-74% of the baseline and an average 51 ± 24% reduction ($P<0.05$). In the control group a 6% increase in the parkinsonian like rotational behavior was measured (Figure 8 and Table 3 summarizes the results).

Table 3

SALINE			CELLS	
RAT	%CHANGE		RAT	% CHANGE
1	1.66		5	0.75
3	0.88		8	0.48
6	1.18		10	0.21
9	0.96		14	0.24
15	0.62		18	0.74
			20	0.64
	saline	cells		
AVG	**1.06**	**0.51**		**0.51**
STD	**0.39**	**0.24**		**0.24**

NOTE: COMPARISON IS FOR 75 MINUTES ONLY.

Altogether the present results support the use of the cells of the present invention to treat Parkinson's disease as was anticipated in Page 59 last paragraph of the instant application.

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